INFECTION AND IMMUNITY, Mar. 2001, p. 1273–1279 Vol. 69, No. 3
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.3.1273–1279.2001

Bacterial Lipopolysaccharide and Tumor Necrosis Factor Alpha Synergistically Increase Expression of Human Endothelial Adhesion Molecules through Activation of NF-κB and p38 Mitogen-Activated Protein Kinase Signaling Pathways

HUBERTUS P. A. JERSMANN,† CHARLES S. T. HII, JUDITH V. FERRANTE, AND ANTONIO FERRANTE*

Department of Immunopathology, The Women's and Children's Hospital, North Adelaide, South Australia 5006, and Department of Paediatrics, University of Adelaide, Adelaide, South Australia 5000, Australia

Received 19 June 2000/Returned for modification 18 September 2000/Accepted 22 November 2000

One of the recognized associations of bacterial infection with cardiovascular events is the activation of endothelium and upregulation of adhesion molecules. The two major proinflammatory mediators implicated in the causation of cardiovascular events, bacterial lipopolysaccharide (LPS) and tumor necrosis factor alpha (TNF), were found to cooperate to enhance the adhesive properties of endothelial cells. These caused synergistic upregulation of intercellular adhesion molecule-1, E-selectin, and vascular cell adhesion molecule-1 in human umbilical vein endothelial cells as determined by flow cytometry analysis and enzyme-linked immunosorbent assay. This synergism was not due to TNF causing an upregulation of CD14 expression. Treatment with both LPS and TNF resulted in a marked increase in the translocation of NF-κB into the nucleus. The activity of p38 mitogen-activated protein kinase was also synergistically enhanced, while the activity of c-*jun* N-terminal kinase was increased in an additive manner. The results demonstrate that LPS and TNF act synergistically to upregulate the expression of endothelial cell adhesion molecules, possibly by amplification of signaling pathways upstream of transcription. These findings have implications for the understanding of the acceleration of atherosclerotic events seen in low-grade infections with gram-negative organisms.

Bacterial lipopolysaccharide (LPS), a major component of the cell wall of gram-negative bacteria, is a highly biologically active molecule which stimulates macrophages to produce and release tumor necrosis factor (TNF) (14). One of the consequences of TNF production is the upregulation of vascular endothelial cell adhesion molecule expression. Apart from this indirect effect on endothelial cells via TNF, LPS per se has the ability to upregulate endothelial cell adhesion molecules directly (3, 32). Endothelial adhesion molecules play an important role in regulating the movement of leukocytes from the blood to foci of inflammation (8, 10, 12).

E-selectin, a member of the selectin family of adhesion molecules, not normally present on the surface of unstimulated endothelial cells, appears on the cell surface within a few hours after exposure to a number of proinflammatory stimuli. Intercellular adhesion molecule 1 (ICAM-1), constitutively expressed at low levels on endothelial cells and vascular cell adhesion molecule-1 (VCAM-1), not expressed on resting endothelial cells, are members of the immunoglobulin superfamily and can be upregulated many fold by proinflammatory mediators including TNF and LPS (3, 4, 9, 20).

In addition to conventional risk factors for coronary events it has been recognized that there is an association between bacterial infection and myocardial infarction (23, 33). Indeed, the addition of inflammatory markers to screening based on lipid levels is a stronger mediator of cardiovascular events (27). Atherosclerotic disease is now viewed as an inflammatory disease (29). In an attempt to investigate the links between infection and vascular disease the gram-negative organism *Chlamydia pneumoniae* has been found to be present in atheromatous lesions (6). It has been demonstrated in vitro that this organism invades and activates endothelial cells (11). The resulting upregulation of endothelial adhesion molecules promotes leukocyte infiltration and atherosclerotic lesions (29). However, the contribution of this organism to ischemic heart disease is still not clear (34).

In infection-induced inflammation both exogenous and endogenous mediators are present, forming a proinflammatory network which is likely to operate by cross talk (13, 15). The manner in which these mediators act within the network remains poorly defined, and we have investigated this concept by studying the relationship between LPS and TNF with respect to expression of the adhesion molecules E-selectin, ICAM-1, and VCAM-1, on endothelial cells.

MATERIALS AND METHODS

Materials. Human recombinant TNF alpha (TNF-α) was a gift from G. R. Adolf, Ernst Boehringer Institute, Vienna, Austria. The activity was 6×10^7 U/mg, and the preparation was >99% pure. The endotoxin contamination was less than 0.125 endotoxin units/ml as assessed by the *Limulus* lysate assay. LPS from *Escherichia coli* O127:B8, chromatographically purified by gel filtration, was purchased from Sigma Chemical Co., St. Louis, Mo.

Mouse anti-human monoclonal antibodies against E-selectin, ICAM-1, and VCAM-1 were purchased from Becton Dickinson. The secondary antibody for

* Corresponding author. Mailing address: Department of Immunopathology, The Women's and Children's Hospital, 72 King William Rd., North Adelaide, South Australia 5006, Australia. Phone: 61 8 8204 7216. Fax: 61 8 8204 6046. E-mail: aferrant@medicine.adelaide.edu.au.

† Present addresses: Respiratory Medicine Unit, Rayne Laboratory, University of Edinburgh Teviot Place, Edinburgh EH8 9AG, United Kingdom.

enzyme-linked immunosorbent assay (ELISA) was a rabbit anti-mouse affinity-isolated, horseradish peroxidase (HRP)-conjugated antibody (DAKO, Glostrup, Denmark), the secondary antibody for fluorescence-activated cell sorting (FACS) was a sheep anti-mouse affinity-isolated, fluorescein isothiocyanate-conjugated antibody (Silenus-AMRAD Biotech). The secondary antibody for the ELISA and Western blotting was a goat anti-rabbit HRP-conjugated, affinity-isolated antibody (Silenus-AMRAD). Antibodies against NF-κB p65 (rabbit anti-human), extracellular signal-related kinase (ERK), and p38 (C-20)-G (polyclonal goat anti-human immunoglobulin G) were purchased from Santa Cruz Biotechnology, Santa Cruz, Calif.

Culture of human umbilical vein endothelial cells (HUVEC). Human umbilical cords were collected immediately after delivery and stored in sterile containers at 4°C for a maximum period of 36 h. The veins were cannulated, washed with Hanks balanced salt solution (HBSS), and filled with collagenase (37°C) (0.4 mg/ml; activity, 219 U/mg; collagenase type II; Worthington). After incubation in a waterbath (37°C for 2 min) the content of each vein was collected. The veins were washed once with HBBS to harvest any remaining cells. Cells from each cord were centrifuged separately (450 × *g* for 5 min); the supernatant was discarded; and the pellet was resuspended in RPMI 1640 supplemented with penicillin (80 U/ml), streptomycin (80 μg/ml), L-glutamine (3.2 mmol/liter) (all from ICN-Flow), and pooled, heat-inactivated human group AB serum (20%). Cells were grown to confluence in 75-cm^2 culture flasks (Corning) which were precoated with 0.2% gelatin (Multicel; TRACE Biosciences, Melbourne, Australia). Endothelial cells were identified by their characteristic monolayer cobblestone appearance and positive staining for factor VIII-related antigen using peroxidase-conjugated rabbit immunoglobulin G antibody to human von Willebrand factor (DAKO) and 3,3′-diaminobenzidine. Only first-passage cells from one cord were used for one experiment. Prior to conducting experiments, cells were harvested from culture flasks with trypsin (0.05 mg/ml) and EDTA (0.02 mg/ml) (both from ICN-Flow). For FACS analysis of adhesion molecule expression, cells were plated at 10^5 cells/well in 24-well plates (2.0-cm^2 wells; Linbro, Flow, McLean, Va.). For estimation of adhesion molecule mRNA levels, cells were plated at 5×10^5 cells/well in six-well plates (9.4-cm^2 wells; Linbro, Flow). For ERK, p38, c-*jun* N-terminal kinase (JNK), and NF-κB assays, cells were plated at 2.5×10^6 cells per dish in tissue culture plates (54-cm^2 dishes; Corning). All plates and dishes were precoated with 0.2% gelatin as described above. Cells were used when confluent, usually 24 to 72 h after plating. Prior to treatment the culture medium was removed from the wells and treatments were added in fresh medium containing 5% human group AB serum.

ELISA. HUVEC (5×10^4/well) were plated into 96-well plates which had been precoated with 0.2% gelatin. The cells reached confluence within 1 to 3 days and were treated in the wells. At the end of any treatment period, the wells were washed twice with warm 0.1% bovine serum albumin (BSA) in HBSS, 0.2 ml per well, and then fixed overnight with glutaraldehyde (0.025%; 20°C; 0.2 ml/well). The monolayers were then washed twice with BSA and incubated with blocking buffer (0.1% BSA in HBSS in 0.1 M glycine; 20°C for 2 h). The ELISA was performed with three washes with 0.2 ml of 0.1% BSA per well between each step. HUVEC were incubated with 50 μl of primary monoclonal antibody per well and 70 μl of secondary HRP-conjugated antibody per well 1 h each at 37°C. Finally, 100 μl of enzyme substrate, consisting of 0.55 mg of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate-(6)] per ml and 0.012% hydrogen peroxide in citrate-phosphate buffer, pH 4.2, was added per well, and color was developed until cell-alone wells gave a standardized absorbance reading at 410 nm (optical density, 0.3 units) using an ELISA plate reader (Dynatech MR 7000).

Flow cytometry. Endothelial cells were treated with various concentrations of agonists and for the times indicated in the figure legends. At the end of the treatment period the cells were washed twice with warm HBSS and then enzymatically detached with 0.35 ml of trypsin-EDTA per well. The cell suspension was collected into FACS tubes (Becton Dickinson), transferred onto ice, washed with 0.5 ml of ISOTONE II solution (4°C; Coulter Electronics), and then resuspended in 50 μl of primary antibody solution (1:100) and incubated on ice for 30 min. After three washes with ISOTONE II (0.5 ml) the secondary antibody was added. After 30 min on ice the cells were washed and fixed with formaldehyde-ISOTONE II (1:33; 0.3 ml). The fluorescence intensity of the cell population was analyzed by flow cytometry on a FACScan (Becton Dickinson). Ten thousand cells per sample were counted, and the data were processed using Lysis II software (Becton Dickinson). The fluorescence values of isotype-matched negative controls were substracted from fluorescence values of the treatments.

Measurement of mRNA. The human E-selectin cDNA probe was a 3.8-kb insert cloned into the *Xho*I site of a pB-SK vector, a gift from J. Gamble, Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science, Adelaide, Australia. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe cocktail was purchased from Clontech. HUVEC were treated as described in the legend of Fig. 2. RNA for the slot blots was isolated by the $RNA_{zol}B$ method (Cinna/Biotrex) by direct addition of 0.4 ml of $RNA_{zol}B$ per 10^6 cells and lysis on the tissue culture plate. Chloroform (50 μl) was added to the lysates, and each tube was vigorously shaken for 15 s and placed on ice for 5 min. After centrifugation (14,000 × *g* for 15 min at 4°C) the upper phase was carefully removed and the RNA was precipitated by the addition of an equal volume of isopropanolol by incubation on ice for 15 min. The RNA was pelleted by centrifugation at 14,000 × *g* (15min, 4°C). The RNA samples were diluted in a solution containing formaldehyde, SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), sodium dodecyl sulfate (SDS), and diethyl-pyrocarbonate and heated (65°C for 5 min). The samples were applied to a Gene Screen Plus nylon membrane using a slot blot vacuum manifold (Hoeffer). The filters were baked (2 h at 80°C), and prehybridization was performed for 16 to 24 h in a solution containing formamide, dextran sulfate, SDS, SSC, Denhardt's solution, and denatured herring sperm DNA (NEN, Du Pont). DNA probes were oligolabeled using [α-^{32}P]dCTP (Amersham) as described previously (17) and used for hybridization. The extent of hybridization was subjected to quantitative analysis by Instant Imager (Packard Instruments). The quantitations for the blots were calculated as the value obtained for the E-selectin probing of that blot divided by the value of its GAPDH reprobing and expressed as a normalized ratio.

Nuclear extracts. Nuclear extracts were prepared as described by others (21) with slight modifications. Briefly, confluent endothelial monolayers were stimulated with diluent, TNF, LPS, or TNF and LPS. After incubation cells were washed twice with cold phosphate-buffered saline (8 ml; 4°C), harvested with a cell scraper, and centrifuged (175 × *g* for 5 min at 4°C). The pellet was resuspended in 1 ml of phosphate-buffered saline cold. Following centrifugation (14,000 × *g* at 4°C for 15 s) the cells were lysed (10 min at 4°C) by adding 80 μl of lysis buffer (pH 7.8) (HEPES [10 mmol/liter], $MgCl_2$ [1.5 mmol/liter], KCl [10 mmol/liter], sucrose [300 mmol/liter 0.5% [vol/vol] Nonidet P-40, dithiothreitol [1 mmol/liter], phenylmethylsulfonyl fluoride [1 mmol/liter], leupeptin [10 μg/ml], aprotinin [10 μg/ml], pepstatin A [10 μg/ml] benzamidine [10 μg/ml]). The tubes were washed a second time, the pellets were resuspended in 60 μl of lysis buffer, and the nuclei were disrupted by sonication. After centrifugation (14,000 × *g* for 30 s) an aliquot was kept aside for protein determination while the remaining supernatants were collected, mixed with Laemmli buffer (100°C for 5 min), and stored at −70°C until analyzed by Western blotting.

Western blotting. The Western blotting assay was carried out as described previously (16). Briefly, equal amounts of nuclear proteins (usually 80 to 100 μg) were separated by SDS–12% polyacrylamide gel electrophoresis (SDS–12% PAGE). After transfer, equal loading per lane was confirmed by staining the intracellular membrane with Ponceau S. The blots were then blocked and incubated with primary antibody, washed (six times), and subsequently incubated with secondary antibody (both antibodies at 1:1,000; 1 h at 37°C). After six washes immunocomplexes were visualized by enhanced chemiluminescence. The blots were scanned with an ImageQuant scanner and quantified with ImageQuant software version 3.3 (Molecular Dynamics, USA).

ERK, p38, and JNK assays. After exposure of HUVEC to the various agonists for 15 min incubations were terminated by removing the culture medium and washing the cells twice (8 ml of HBSS at 4°C). Per dish, 200 μl of lysis buffer (HEPES [20 mmol/liter] [pH 7.4], 0.5% [vol/vol] Nonidet P-40, NaCl [100 mmol/liter], EDTA [1 mmol/liter], Na_3VO_4 [2 mmol/liter], dithiothreitol [2 mmol/liter], phenylmethyl sulfonyl fluoride [1 mmol/liter], leupeptin [10 μg/ml], aprotinin [10 μg/ml], pepstatin A [10 μg/ml], benzamidine [10 μg/ml]) was added, and the cells were harvested with a cell scraper and incubated with constant mixing (4°C for 2 h). After centrifugation (14,000 × *g* for 20 s) an aliquot of each supernatant was taken for protein determination while the remaining supernatants were collected and stored at −70°C until assayed for kinase activity. Equal amounts of ERK and p38 were immunoprecipitated before determination of kinase activity as described elsewhere (16). Briefly, lysates (usually 800 μg of protein) were precleared with protein A-Sepharose. Anti-p38 or anti-ERK antibody (3 μg/sample) was added, and tubes were incubated with constant mixing (90 min at 4°C). The antigen-antibody complexes were precipitated by the addition of protein A-Sepharose. The immunoprecipitates were washed twice at 4°C, first with lysis buffer and then with assay buffer (HEPES [20 mmol/liter] [pH 7.2], β-glycerophosphate [20 mmol/liter], *p*-nitrophenylphosphate [3.8 mmol/liter], $MgCl_2$ [10 mmol/liter], dithiothreitol [1 mmol/liter], Na_3VO_4 [50 μmol/liter], ATP [20 μmol/liter]). The assay was started by adding 30 μl of assay buffer containing 6 μCi of [γ-^{32}P]ATP per sample and 35 μg of myelin basic protein per ml. After a 20-min incubation at 30°C, the assay was terminated by the addition of Laemmli buffer and the samples were boiled (100°C for 5 min). Phosphorylated myelin basic protein was resolved by SDS–16% PAGE and was detected and quantified using an Instant Imager and Imager software (Packard Instruments). JNK was assayed by a solid-phase method as described previously (16). Briefly,

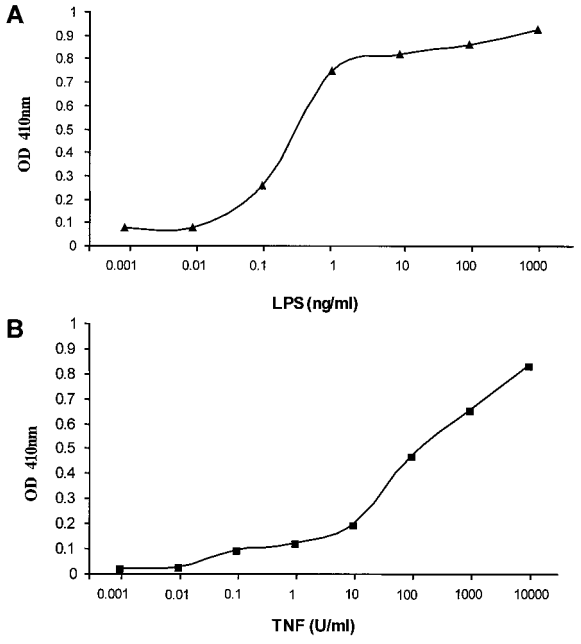

FIG. 1. The effect of varying the concentration of LPS and TNF-α on the induction of E-selectin expression in endothelial cells. HUVEC (5×10^4 cells/well) were stimulated for 6 h with the indicated concentrations of LPS (A) or TNF-α (B) in the presence of serum (10%). E-selectin was measured by ELISA. The results show the means of six determinations from two experiments with cells from two different cords. The means of untreated cells were 0.007 (A) and 0.02 (B) and have been subtracted from the LPS and TNF-α treatment results. OD, optical density.

glutathione *S*-transferase-Jun (1–79) fusion protein was purified from bacterial lysates using glutathione-Sepharose 4B (Pharmacia Biotech) at 4°C with gentle rocking. Lysate protein (1,000 μg), $MgCl_2$ (15 mmol/liter), and ATP (10 μmol/liter) were added to 25 μl (packed volume) of glutathione *S*-transferase-Jun (1–79) coupled to glutathione-Sepharose beads. The mixtures were incubated with gentle rocking (2 h at 4°C). After centrifugation (14,000 × *g* at 4°C for 5 min) the beads were washed once with lysis buffer, once with wash buffer (pH 7.0) [piperazine-*N, N*′-bis(2-ethanesulfonic acid) (10 mmol/liter), NaCl (100 mmol/liter)] and once with assay buffer (as above). The assay was started by adding 30 μl of assay buffer containing 6 μCi of [γ-^{32}P]ATP/sample (30°C for 20 min) and terminated by the addition of Laemmli buffer (100°C for 5 min). Samples were resolved by SDS–12% PAGE, and detection and quantification of phosphorylated glutathione *S*-transferase-Jun (amino acid residue 1–79) were carried out as above.

Statistical analysis. Data were processed by either analysis of variance or the nonparametric Kruskal-Wallis test with Dunne's posttest if the data were not normally distributed.

RESULTS

TNF and LPS act synergistically to upregulate HUVEC adhesion molecule expression. Concentration-response studies were performed to determine the lowest concentrations of TNF and LPS which produced a response. These were found to be 5 U of TNF per 10^5 cells and 0.1 ng of LPS per ml (Fig. 1). Consistent with previous studies our results demonstrated that E-selectin began to appear on the cell surface 2.5 to 3 h following TNF or LPS addition and peaked at 6 h. VCAM-1 expression was evident at 7 to 8 h and was maximal at 12 h. ICAM-1 was constitutively expressed on resting cells, and its upregulation followed a similar time course to that of VCAM-1 (Fig. 2).

To examine whether the presence of one proinflammatory mediator would alter the responses of endothelial cells to another mediator, endothelial cell monolayers were treated with diluent, TNF (5 U/10^5 cells), LPS (0.1 ng/ml), or TNF and LPS, and the expression of E-selectin, ICAM-1, and VCAM-1 was assessed by ELISA and indirect immunofluorescence staining and flow cytometry. While there was little or no upregulation of expression adhesion molecule in the presence of TNF or LPS individually, the combined presence of TNF and LPS exerted a synergistic effect on the expression of adhesion molecules (Fig. 3 to 5). For E-selectin the synergy between TNF and LPS was observed at 3, 4, and 6 h; for ICAM-1 it was observed at 7.5, 9.5, and 11.5 h; and for VCAM-1 it was observed at 9.5 and 11.5 h.

To examine whether the combined effect of TNF and LPS observed was also reflected at the level of adhesion molecule mRNA expression, the effect of TNF and LPS on the induction of E-selectin mRNA was measured after 2 h of stimulation. The treatment with TNF and LPS resulted in a significantly greater increase in E-selectin mRNA levels than those increases produced by each agonist alone, and this increase was also greater than the sum of the responses caused by TNF and LPS alone (Fig. 6).

Effects on NF-κB accumulation in the nucleus. NF-κB is activated by proinflammatory cytokines and bacterial products. Activation is achieved by the phosphorylation and subsequent degradation of NF-κB's naturally occurring cytosolic inhibitor, IκB. p65 rather than p50 was examined because it is the dominant, transcriptionally active subunit of NF-κB (30). The activated NF-κB then translocates into the nucleus, where it

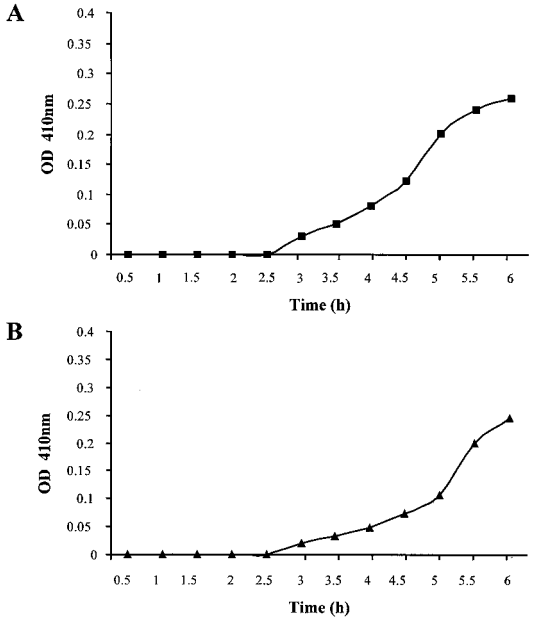

FIG. 2. Time course of the induction of E-selectin expression on the surface of endothelial cells by TNF-α and LPS. HUVEC were grown to confluence in 96-well plates (5×10^4 cells/well) and treated with either TNF-α (5 U/ml) (A) or LPS (0.1 ng/ml) (B) for the indicated time periods. E-selectin was measured by ELISA. The results are the means of five determinations from two experiments with cells from two different cords. The values for unstimulated cells were 0.01 and have been subtracted from the treatment results. OD, optical density.

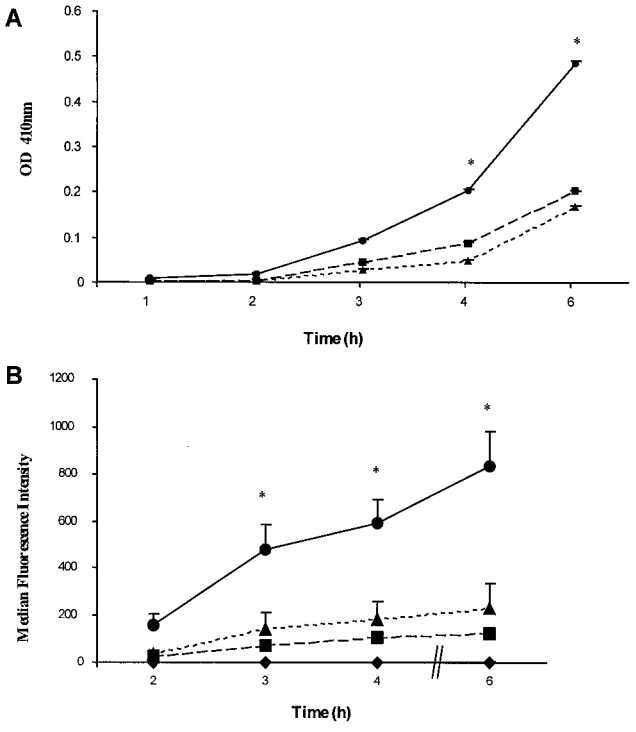

FIG. 3. Effect of combined TNF-α and LPS treatment on E-selectin expression by endothelial cells. HUVEC (5×10^4 cells/well) were treated with either TNF (5 U/well) (■) or LPS (0.1 ng/ml), (▲), or TNF plus LPS (●) for the times indicated. The results are the means ± standard errors of the means (error bars) of five experiments with cells from five cords. (A) E-selectin was measured by ELISA. The value for untreated cells was 0.009 and was subtracted from the treatment results. Each experiment was run in triplicate. *, for TNF versus TNF plus LPS or LPS versus TNF plus LPS, $P < 0.001$; for the sum of TNF and LPS versus TNF plus LPS, $P < 0.01$. OD, optical density. (B) E-selectin was measured by flow cytometry. Cells were treated with diluent (◆), TNF (5 U/10^5 cells) (■), LPS (0.1 ng/ml) (▲) or TNF plus LPS (●) for the times indicated. The mean fluorescence intensity of 10,000 cells per sample was determined. *, for TNF or LPS versus TNF plus LPS, $P < 0.001$ at 3, 4, and 6 h; for the sum of TNF and LPS versus costimulation (TNF plus LPS), $P < 0.001$ at 3, 4, and 6 h.

binds to the promoters of adhesion molecule genes (21, 25). This is a transient phenomenon that peaks between 1 and 4 h after a stimulus and diminishes thereafter, coinciding with the reconstitution of IκB (26). Experiments were set up in which endothelial cells were treated with diluent, TNF, LPS, or TNF and LPS for 2.5 h and the quantity of NF-κB in nuclear extracts was assayed by Western blotting. The amount of NF-κB that had accumulated in the nucleus after the combined treatment with TNF and LPS was significantly greater than that obtained with either TNF or LPS alone, as well as greater than the sum of these responses (Fig. 7). When nuclear extracts of HUVEC were assayed after 2.5, 8, 20, or 28 h of incubation in the presence of both TNF and LPS, there was still an increase in NF-κB after 28 h (Fig. 8).

Effects of TNF and LPS on MAP kinases. Besides NF-κB, the transcriptional factors c-Jun and ATF2 are also required for E-selectin transcription. The transcriptional activities of c-Jun and ATF2 are regulated via their phosphorylation by JNK and/or p38 mitogen-activated protein (MAP) kinase (20, 26). JNK, p38, and ERK are members of three distinct MAP kinase cascades, each consisting of a series of kinases in which upstream kinases activate downstream kinases by phosphorylation (31). While there is no conclusive evidence that ERK is important in TNF-mediated endothelial cell activation, studies have demonstrated that inhibition of the activities of kinases within the JNK and/or p38 cascades results in the inhibition of the transcriptional activity of the E-selectin promoter in endothelial cells (20). LPS also has the capability to stimulate the activities of p38, JNK, and ERK (1, 27).

To examine whether the synergistic stimulation of the expression of E-selectin expression by TNF and LPS was accompanied by similar increases in the activities of these kinases, cells were stimulated with diluent, TNF, LPS, or TNF and LPS at concentrations as stipulated in the legend of Fig. 9, and the activities of MAP kinases were determined. Previous studies showed that maximal activation of ERK, p38, and JNK occurs at 15 min after the stimulation of endothelial cells with either TNF or LPS (26). Costimulation of endothelial cells with TNF and LPS resulted in a synergistic increase in the activity of p38 (Fig. 9A), whereas the activity of JNK was increased only in an additive manner (Fig. 5B). In contrast no synergistic increase in ERK activity was observed (Fig. 9C). There was also no synergism even when the concentrations of TNF and LPS were increased 100-fold (data not shown). These findings are in

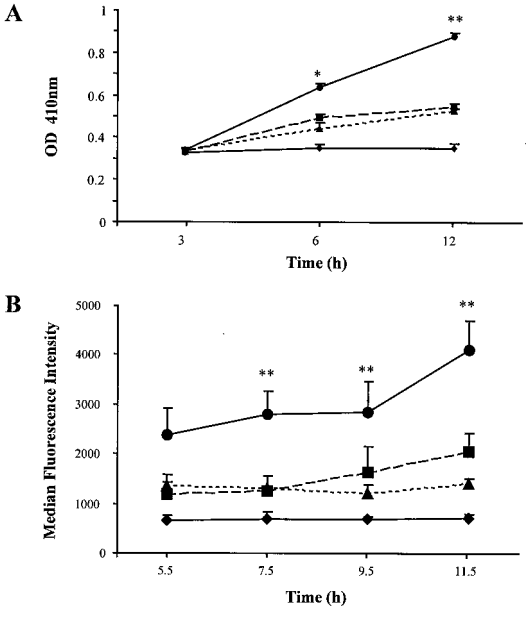

FIG. 4. Effect of combined TNF-α and LPS treatment on ICAM-1 expression by endothelial cells. Treatment conditions were as outlined in the legend of Fig. 3. The data are the means ± standard error of the means (error bars) of four experiments, each conducted with cells from four different cords and each in triplicate. Symbols: ◆, cells treated with diluent only; ■, TNF; ▲ LPS, ●, TNF plus LPS. (A) The expression of ICAM-1 for cells treated for the times indicated was measured by ELISA. *, $P < 0.01$ for TNF versus TNF plus LPS, or LPS versus TNF plus LPS; **, $P < 0.001$ for TNF versus TNF plus LPS or LPS versus TNF plus LPS or $P < 0.01$ for the sum of TNF and LPS versus TNF plus LPS. OD, optical density. (B) Expression of ICAM-1 measured by flow cytometry. **, $P > 0.05$ at 5.5 h, $P < 0.001$ at 7.5 h, $P < 0.01$ at 9.5 h, and $P < 0.001$ at 11.5 h. For the sum of TNF and LPS versus costimulation [TNF plus LPS], P was <0.01 at 7.5, 9.5, and 11.5 h.

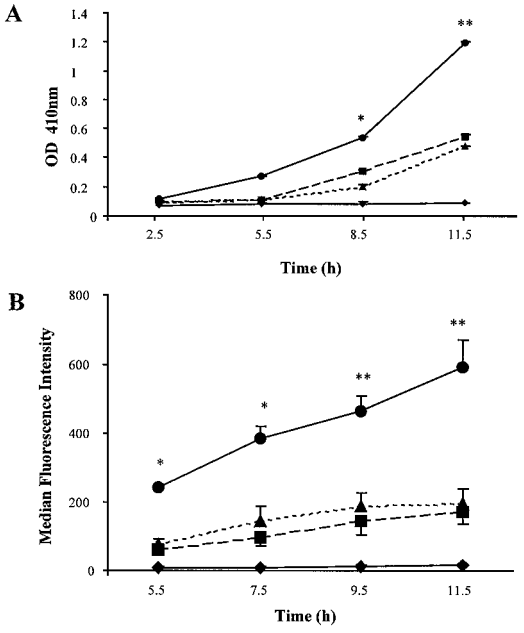

FIG. 5. Effect of combined TNF-α and LPS treatment on VCAM-1 expression by endothelial cells. Treatment conditions were as outlined in the legend of Fig. 3. The data presented are the means ± standard errors of the means (error bars) of four experiments, each conducted with cells from four different cords and each in triplicate. Symbols: ◆ cells treated with diluent only; ■, TNF; ▲, LPS; ●, TNF plus LPS. (A) The expression of VCAM-1 in cells treated for the times indicated was measured by ELISA. *, $P < 0.01$ for TNF versus TNF plus LPS or LPS versus TNF plus LPS; **, $P < 0.001$ for TNF versus TNF plus LPS or LPS versus TNF plus LPS; $P < 0.01$ for the sum of TNF and LPS versus TNF plus LPS (analysis of variance). OD, optical density. (B) The expression of VCAM-1 was measured by flow cytometry (for TNF or LPS versus TNF and LPS, $P < 0.01$ at 5.5, 7.5, and 9.5 h and $P < 0.001$ at 11.5 h; for the sum of TNF and LPS versus costimulation [TNF plus LPS], $P < 0.05$ at 9.5 h and $P < 0.01$ at 11.5 h).

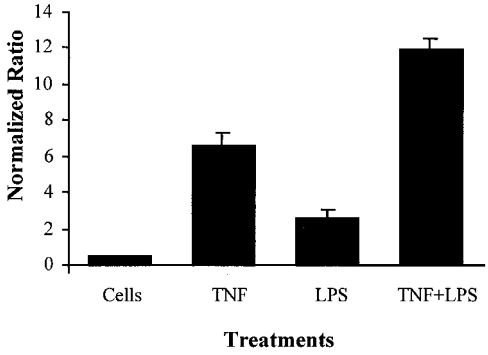

FIG. 6. Effects of combined treatment with TNF and LPS on E-selectin mRNA. E-selectin mRNA was assayed as described in Material and Methods. HUVEC monolayers were treated with either diluent (Cells), TNF (5 U/10^5 cells), LPS (0.1 ng/ml), or TNF plus LPS for a duration of 2 h. The columns represent the means (error bars, standard errors of the means) of three tests with cells from three different cords run at the same time and are expressed as normalized ratios (as described in Materials and Methods). When this experiment was repeated at a different time, similar results and the same statistical significance were found (for TNF versus TNF + LPS, $P < 0.01$; for LPS versus TNF + LPS, $P < 0.001$; for the sum of TNF and LPS versus costimulation [TNF + LPS], $P < 0.01$).

concordance with other studies, which demonstrate that the ERK cascade does not play a major role in endothelial cell activation by either TNF or LPS (26).

DISCUSSION

The data demonstrate that TNF and LPS act synergistically on endothelial cells to increase the adhesive properties of these cells. When added together at concentrations encountered in bacterium-induced inflammation, these mediators caused significantly greater expression of the adhesion molecules E-selectin, ICAM-1, and VCAM-1 than the sum of either alone. Importantly, at these concentrations TNF or LPS per se caused little or no upregulation of endothelial adhesion molecule expression.

The induction of endothelial adhesion molecules is an important early step in the development of an inflammatory reaction; thus, the synergism described is likely to be relevant in immunity to bacteria. Promoting the infiltration of circulating leukocytes to foci of infection may enhance the resolution of an infection; however, promoting the infiltration of leukocytes into atherosclerotic plaques is likely to contribute to an exacerbation of such lesions and possibly to the initiation cardiovascular events. E-selectin and VCAM-1 are instrumental in recruiting monocytes and lymphocytes, which are implicated in atheromatous lesions (28).

Our findings of synergism between mediators demonstrate the importance of cross talk in the regulation of the inflammatory response. To understand how this interaction may operate, we focused on the effects of TNF and LPS on E-selectin mRNA expression since the intracellular signaling pathways that regulate E-selectin expression are known. Consistent with the data on E-selectin protein expression, TNF and LPS were synergistic in upregulating the expression of E-selectin mRNA.

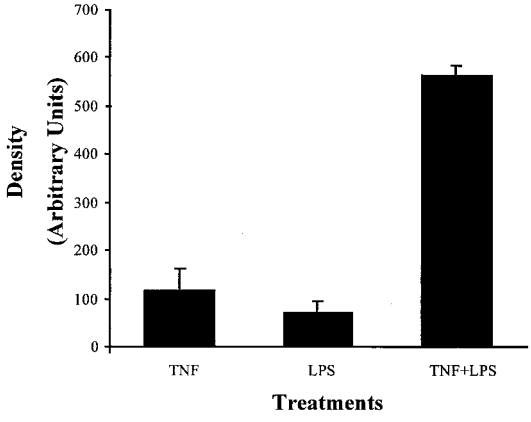

FIG. 7. Effect of TNF and LPS on NF-κB. The amount of NF-κB that was present in the nuclear extracts of either untreated endothelial cells or cells treated with TNF (5 U/10^5 cells), LPS (0.1 ng/ml), or both combined for 2.5 h was measured by Western blotting. The densities of the bands were quantified by ImageQuant 3.3 and are expressed as arbitrary units. The data are means + standard errors of the means (error bars) derived of three experiments, each with cells from a different cord at a different time. The mean of the values of cells treated with diluent (38 arbitrary units) has been subtracted from all other treatment results. For the sum of TNF and LPS versus costimulation (TNF + LPS), $P < 0.001$.

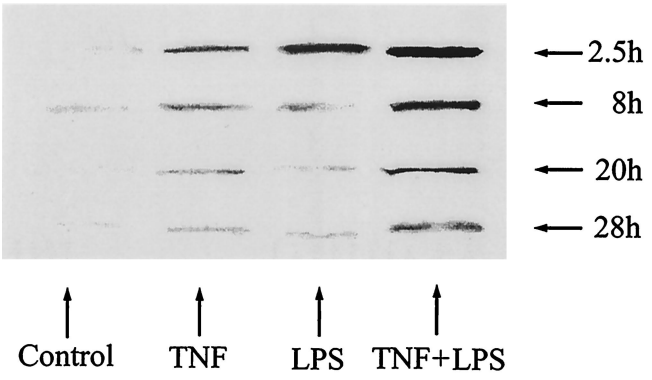

FIG. 8. Time course of NF-κB activation in HUVEC. Cells were harvested and processed at 2.5, 8, 20, and 28 h. The amount of NF-κB in nuclear extracts was still upregulated at 28 h. Shown are scans of radiographic bands obtained with a Molecular Dynamics Densitometer (model 1.30) and ImageQuant Scanner software (version 3.3).

TNF and LPS individually have been shown to activate NF-κB in endothelial cells (20), and our data demonstrate that TNF and LPS caused a synergistic activation of NF-κB. Although the precise mechanism by which TNF and LPS caused the synergistic activation of NF-κB is not known, it is possible that the signaling cascade due to TNF and LPS converged at NF-κB-inducing kinase. Consistent with this synergism, it has been shown independently that TRAF2 (activated by TNF) and TRAF6 (activated by LPS) directly interact with NF-κB-inducing kinase (23, 24).

The importance of the NF-κB system for endothelial adhesion molecule expression is illustrated by the fact that overexpression of IκB blocks TNF-induced VCAM-1 transcriptional activity (35), inhibition of NF-κB reduces E-selectin expression (5), and overexpression of the p65 subunit of NF-κB results in activation of E-selectin promoter activity without stimuli (7). The observation that in the presence of both TNF and LPS the usually transient activation of NF-κB persisted for as long as 28 h is consistent with the role played by this transcription factor in the amplification and perpetuation of the inflammatory response (2). Furthermore, activated NF-κB has been found to be present in atherosclerotic lesions (5).

When the activities of other relevant intracellular signaling molecules, p38, JNK, and ERK, were investigated, TNF and LPS were shown to be synergistic in the upregulation of the activity of p38. The activity of JNK was increased in an additive manner. While both mediators individually increased the activity of ERK, there was no enhancement with costimulation. These data suggest that NF-κB, and possibly p38, and JNK but not ERK are likely to contribute to the TNF and LPS synergism on the upregulation of E-selectin expression.

Recently we have demonstrated the presence of the LPS receptor, CD14, on HUVEC (17a). Previously we also demonstrated that TNF upregulated CD14 expression on human neutrophils (18). However, it is unlikely that this is a mechanism of synergism between the cytokine and LPS in HUVEC since we found that treatment of HUVEC with 10 to 100 U of TNF did not increase the expression of CD14 (data not presented).

During infection immune cells as well as other cell types are exposed concomitantly to a variety of mediators, including

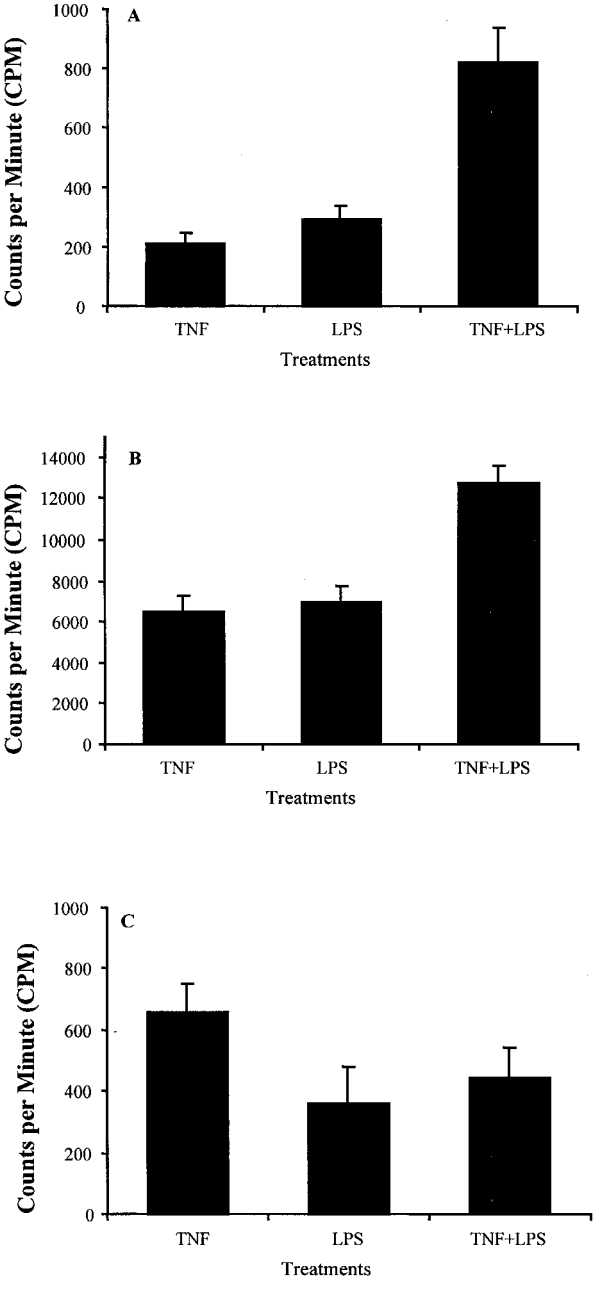

FIG. 9. The effects of TNF and LPS on the activities of p38 (A), JNK (B), and ERK (C) in HUVEC stimulated with either diluent, TNF (5 U/ 10^5 cells), LPS (0.1 ng/ml), or TNF and LPS combined. The cells were treated for 15 min and then lysed and processed as described in Materials and Methods. The results for p38 and ERK represent counts per minute of ^{32}P incorporated in myelin basic protein; the results for JNK represent counts per minute of ^{32}P incorporated in glutathione *S*-transferase-Jun (1–79). The means + standard errors of the means (error bars) of five experiments, each conducted with cells from a different cord, are shown. (A) p38. The baseline of 1,306 cpm for diluent-treated cells has been subtracted from the mean results of the other treatments. For each TNF or LPS versus TNF + LPS, $P < 0.01$; for the sum of TNF and LPS versus costimulation (TNF + LPS), $P < 0.05$. (B) JNK. The baseline of 520 cpm was subtracted from the mean results of the other treatments. For each TNF or LPS versus TNF + LPS, $P < 0.001$; for the sum of TNF and LPS versus costimulation (TNF + LPS), $P > 0.05$. (C) ERK. The baseline of 1,327 cpm was subtracted from the mean results of the other treatments. There were no statistically significant differences between treatments.

cytokines and bacterial products. Synergism between mediators as demonstrated by us for TNF and LPS in terms of endothelial adhesion molecule expression may influence the course of bacterial infection and endothelial pathophysiology such as atherosclerosis. In addition to TNF, LPS is capable of inducing a range of inflammatory mediators such as interleukin 1 and activating complement components. It would be interesting to compare other mediators with the effects of TNF on the LPS responses, taking into consideration those mediators which use intracellular signaling molecules (e.g., interleukin 1) (36) very similar to and those which use signaling pathways (complement) significantly different from LPS. The understanding of these interactions and their mechanisms is pivotal to the development of new strategies to manipulate the intercellular mediator network for therapeutic advantages.

ACKNOWLEDGMENTS

We are indebted to the birthing mothers, who donated their cords, and the midwives of the labor ward of the Women's and Children's Hospital, North Adelaide, Australia, who provided them to us 24 h a day. We are grateful to Ian Bates from the Red Cross, Adelaide, South Australia, Australia, for the generous supply of human group AB serum.

This work received support from the National Heart Foundation of Australia and the National Health and Medical Research Council of Australia.

REFERENCES

1. **Arditi, M., J. Zhou, M. Torres, D. L. Durden, M. Stins, and S. K. Kwang.** 1995. Lipopolysaccharide stimulates the tyrosine phosphorylation of mitogen-activated protein kinases p44, p42 and p41 in vascular endothelial cells in a soluble CD14-dependent manner. J. Immunol. **155:**3994–4003.
2. **Barnes, P. J., and M. Karin.** 1997. Nuclear factor-κB - a pivotal transcription factor in chronic inflammatory diseases. N. Engl. J. Med. **336:**1066–1071.
3. **Beekhuizen, H., I. Blokland, A. J. Corsel-van Tilburg, F. Koning, and R. van Furth.** 1991. CD14 contributes to the adherence of human monocytes to cytokine-stimulated endothelial cells. J. Immunol. **147:**3761–3767.
4. **Bevilacqua, M. P., R. M. Nelson, G. Mannori, and O. Cecconi.** 1994. Endothelial-leukocyte adhesion molecules in human disease. Annu. Rev. Med. **45:**361–378.
5. **Brand, K., S. Page, G. Rogler, A. Bartsch, R. Brandl, R. Knuechel, M. Page, C. Kaltschmidt, P. A. Baeuerle, and D. Neumeier.** 1996. Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. J. Clin. Investig. **97:**1715–1722.
6. **Campbell, L. A., C.-C. Kuo, and J. T. Grayston.** 1998. *Chlamydia pneumoniae* and cardiovascular disease. Emerg. Infect. Dis. **4:**571–579.
7. **Chen, C. C., and A. M. Manning.** 1995. Transcriptional regulation of endothelial cell adhesion molecules: a dominant role for NF-κB. Agents Actions Suppl. **47:**135–141.
8. **Fast, D. J., P. M. Schlievert, and R. D. Nelson.** 1989. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. Infect. Immun. **57:**291–294.
9. **Faull, R. J.** 1995. Adhesion molecules in health and disease. Aust. N. Z. J. Med. **25:**720–730.
10. **Frenette, P. S., and D. D. Wagner.** 1996. Adhesion molecules-part II: Blood vessels and blood cells. N. Engl. J. Med. **335:**43–45.
11. **Fryer, R. H., E. P. Schwobe, M. L. Woods, and G. M. Rodgers.** 1997. Chlamydia species infect human vascular endothelial cells and induce procoagulant activity. J. Investig. Med. **45:**168–174.
12. **Gamble, J. R., J. M. Harlan, S. J. Klebanoff, and M. A. Vadas.** 1985. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. Proc. Natl. Acad. Sci. USA **82:**8667–8671.
13. **Ginsburg, I., and R. Kohen.** 1995. Cell damage in inflammatory and infectious sites might involve a coordinated "cross-talk" among oxidants, microbial haemolysins and ampiphiles, cationic proteins, phospholipases, fatty acids, proteinases and cytokines (an overview). Free Rad. Res. **22:**489–517.
14. **Haraldsen, G., D. Kvale, B. Lien, I. N. Farstad, and P. Brandtzaeg.** 1996. Cytokine-regulated expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in human intestinal microvascular endothelial cells. J. Immunol. **156:**2558–2565.
15. **Henderson, B., S. Poole, and M. Wilson.** 1996. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. **60:**316–341.
16. **Hii, C. S. T., Z. H. Huang, A. Bilney, M. Costabile, A. W. Murray, D. A. Rathjen, C. J. Der, and A. Ferrante.** 1998. Stimulation of p38 phosphorylation/activation by arachidonic acid in HeLa cells, HL60 promyelocytic leukaemic cells and human neutrophils: evidence for cell-type specific activation of MAP kinases. J. Biol. Chem. **273:**19277–19282.
17. **Huang, Z. H., E. J. Bates, J. V. Ferrante, A. Poulos, B. S. Robinson, and A. Ferrante.** 1997. Inhibition of stimuli-induced endothelial cell intercellular adhesion molecule-1, E-selectin and vascular cellular adhesion molecule-1 expression by arachidonic acid and its hydroxy- and hydroperoxy-derivatives. Circ. Res. **80:**149–158.

17a. **Jersmann, H. P. A., C. S. T. Hii, G. L. Hodge, and A. Ferrante.** 2001. Synthesis and surface expression of CD14 by human endothelial cells. Infect. Immun. **69:**479–485.

18. **Jersmann, H. P. A., D. A. Rathjen, and A. Ferrante.** 1998. Enhancement of lipopolysaccharide-induced neutrophil oxygen radical production by tumor necrosis factor alpha Infect. Immun. **66:**1744–1747.
19. **Khachigian, L. M., J. W. U. Fries, M. W. Benz, D. T. Bonthron, and T. Collins.** 1994. Novel *cis*-acting elements in the human platelet-derived growth factor B-chain core promoter that mediate gene expression in cultured vascular endothelial cells. J. Biol. Chem. **269:**22647–22656.
20. **Min, W., and J. Pober.** 1997. TNF initiates E-selectin transcription in human endothelial cells through parallel TRAF-NF-κB and TRAF-rac/CDC42-JNK-c-JUN/ATF2 pathways. J. Immunol. **159:**3508–3518.
21. **Neish, A. S., L. M. Khachigian, A. Park, V. R. Baichwal, and T. Collins.** 1995. Sp1 is a component of the cytokine-inducible enhancer in the promoter of vascular cell adhesion molecule-1. J. Biol. Chem. **270:**28903–28909.
22. **Nieminen, M. S., K. Mattila, and V. Valtonen.** 1993. Infection and inflammation as risk factors for myocardial infarction. Eur. Heart J. **14**(Suppl. K)**:**12–16.
23. **O'Neill, L. A. J., and C. Greene.** 1998. Signal transduction pathways activated by the IL-1 receptor family: ancient signalling machinery in mammals, insects and plants. J. Leukoc. Biol. **63:**650–657.
24. **Pober, J. S.** 1998. Activation and injury of endothelial cells by cytokines. Pathol. Biol. Paris **46:**159–163.
25. **Read, M. A., M. Z. Whitley, A. J. Williams, and T. Collins.** 1994. NF-κB and IκBα: an inducible regulatory system in endothelial activation. J. Exp. Med. **179:**503–512.
26. **Read, M. A., M. Z. Whitley, S. Gupta, J. W. Pierce, J. Best, R. J. Davis, and T. Collins.** 1997. Tumor necrosis factor α-induced E-selectin expression is activated by the nuclear factor-κB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. J. Biol. Chem. **272:**2753–2761.
27. **Ridker, P. M., C. H. Hennekens, J. E. Burning, and N. Rifai.** 2000. C-rective protein and other markers of inflammation in the prediction of cardiovascular diseases in women. N. Engl. J. Med. **342:**836–843.
28. **Ross, R.** 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature **362:**801–809.
29. **Ross, R.** 1999. Atherosclerosis-an inflammatory disease. N. Engl. J. Med. **340:**115–126.
30. **Schmitz, M. L., M. A. dos Santos Silva, and P. A. Baeuerle.** 1995. Transactivation domain (TA_2) of p65 NF-κB. J. Biol. Chem. **270:**15576–15584.
31. **Schulze-Osthoff, K., D. Ferrari, K. Riehemann, and S. Wesselborg.** 1997. Regulation of NF-κB activation by MAP kinase cascades. Immunobiology **198:**35–49.
32. **Schumann, R. R., D. Pfeil, N. Lamping, C. Kirschning, G. Scherzinger, P. Schlag, L. Karawajew, and F. Herrmann.** 1996. Lipopolysaccharide induces the rapid tyrosine phosphorylation of the mitogen-activated protein kinases erk-1 and p38 in cultured human vascular endothelial cells requiring the presence of soluble CD14. Blood **87:**2805–2814.
33. **Sessa, R., M. I. Di Pietro, I. Santino, M. del-Piano, A. Varveri, A. Dagianti, and M. Penco.** 1999. *Chlamydia pneumoniae* infection and atheroslerotic coronary disease. Am. Heart J. **137:**1116–1119.
34. **Wald, N. J., M. R. Law, J. K. Morris, X. Zhou, and M. E. Ward.** 2000. *Chalamydia pneumoniae* infection and mortality from ischaemic heart disease: large prospective study. Br. Med. J. **321:**204–207.
35. **Weber, K. S., G. Draude, W. Erl, and C. Weber.** 1999. Monocyte arrest and transmigration on inflamed endothelium in shear flow is inhibited by adenovirus-mediated gene transfer of IkappaB-alpha. Blood **93:**3685–3693.
36. **Zhang, F. X., C. J. Kirshing, R. Mancinelli, X.-P. Xu, Y. Jin, E. Faure, A. Montovani, M. Rother, M. Muzio, and M. Arditi.** 1999. Bacterial lipopolysaccharide activates nuclear factor KB through interleuking-1 signalling mediators in cultures of human dermal endothelial cells and mononuclear phagocytes. J. Biol. Chem. **274:**7611–7614.

Editor: R. N. Moore